Ecological impact assessment of a bioaugmentation site on remediation of chlorinated ethylenes by multi-omics analysis

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Summary

Bioremediation may affect the ecological system around bioremediation sites. However, little is known about how microbial community structures change over time after the initial injection of degraders. In this study, we have assessed the ecological impact of bioaugmentation using metagenomic and metatranscriptomic approaches to remove trichlorinated ethylene/cis-dichloroethylene (TCE/cDCE) by *Rhodococcus jostii* strain RHA1 as an aerobic chemical compound degrader. Metagenomic analysis showed that the number of organisms belonging to the genus *Rhodococcus*, including strain RHA1, increased from 0.1% to 76.6% of the total microbial community on day 0 at the injection site. Subsequently, the populations of strain RHA1 and other TCE/cDCE-degrading bacteria gradually decreased over time, whereas the populations of the anaerobic dechlorinators *Geobacter* and *Dehalococcoides* increased at later stages. Metatranscriptomic analysis revealed a high expression of aromatic compound-degrading genes (*bphA1-A4*) in strain RHA1 after RHA1 injection. From these results, we concluded that the key dechlorinators of TCE/cDCE were mainly aerobic bacteria, such as RHA1, until day 1, after which the key dechlorinators changed to anaerobic bacteria, such as *Geobacter* and *Dehalococcoides*, after day 6 at the injection well. Based on the α-diversity, the richness levels of the microbial community were increased after injection of strain RHA1, and the microbial community composition had not been restored to that of the original composition during the 19 days after treatment. These results provide insights into the assessment of the ecological impact and bioaugmentation process of RHA1 at bioremediation sites.

Keywords: bioremediation; chlorinated ethylenes; next-generation sequencing; metatranscriptomics; metagenomics; *Rhodococcus jostii* RHA1
INTRODUCTION

Chlorinated solvents, such as trichloroethylene (TCE) and tetrachloroethylene (PCE), are widely used in industrial processes and the agricultural industry. These compounds have been used in vapor-degreasing, paint removers, metal cleaning, and dry-cleaning/wool facilities (Suttinun et al., 2013). However, chlorinated solvents, including cis-dichloroethylene (cDCE) and vinyl chloride (VC), which are produced as intermediates of TCE degradation in the environment, have caused serious environmental problems. Chlorinated solvents tend to accumulate and diffuse at the bottom of aquifers originating from dense nonaqueous-phase liquids (DNAPLs), and can provide a persistent source of contamination for decades. Moreover, TCE induces various diseases, such as cancer, in animals and humans (Adetutu et al., 2015; Eder, 1991; Gilbert et al., 2009; Rusyn et al., 2014).

A number of physical and chemical approaches, such as excavation, in situ chemical oxidation with permanganate, and in situ thermal desorption, can be used for the removal of pollutants from groundwater and soil (Crane and Scott, 2012; Heron et al., 2013; Perelo, 2010). However, these approaches are inadequate for the removal of chlorinated solvents, which tend to spread in the environment at low concentrations. Microbes can be used effectively to treat TCE-contaminated environments at a low cost and to degrade TCE/cDCE completely to produce nontoxic compounds, such as ethane, glyoxylic acid, and formic acid.

Bioaugmentation is a bioremediation method that reduces contamination at certain sites by the introduction of specific bacteria, or a consortium of bacteria (Duba et al., 1996). Bioaugmentation is a short-term remedy compared with biostimulation; therefore, this method is thought to be a useful clean-up approach at bioremediation sites (Tiehm and Schmidt, 2011). For bioaugmentation, both anaerobic and aerobic microorganisms can be used as degraders for contaminated substrates. The anaerobic reductive dechlorination (RD) of chlorinated solvents
can be mediated by anaerobic microorganisms such as *Dehalobacter*, *Dehalococcoides*, *Desulfitobacterium*, *Desulfuromonas*, *Dehalogenimonas*, *Geobacter*, and *Sulfurospirillum* (Frascari et al., 2015). These bacteria have been applied to remediate TCE and their derivatives at sites of contamination. In a previous study, cultured strain KB-1 was injected into TCE-contaminated groundwater to degrade TCE to ethane in Southern Ontario, Canada (Pérez-de-Mora et al., 2014). Another study reported that *Dehalococcoides* strains FL2, BAV1, and GT with nutrients were able to transform 100% of TCE within 17 weeks at the injection site (Patil et al., 2014). In addition, Adetutu et al. (2015) reported that bioaugmentation of TCE affects aquifers with *Dehalococcoides* by the RD approach at field sites. Furthermore, another study reported the effects of bioaugmentation with a mixed *Dehalococcoides/Dehalobacter* culture for the treatment of 1,1,1-trichloroethane (TCA) and chloroethenes in groundwater at three Danish sites (Scheutz et al., 2014).

Although TCE-degrading microorganisms are useful tools for in situ field-scale bioaugmentation, anaerobic microorganisms of these bacteria convert TCE/cDCE into VC as the intermediate, which is harmful to humans, during the reductive dechlorination reaction (Chambon et al., 2013; Kielhorn et al., 2000). In addition, the degradation velocity of VC is rate-limiting; thus, VC persists in the bioremediation site for a long time. In contrast, aerobic bacteria do not convert TCE to VC as the intermediate because these bacteria degrade TCE/cDCE via the epoxidation of short-chain alkenes by an alkene monooxygenase (Suttinun et al., 2013). Therefore, aerobic microorganisms are a more attractive choice for bioaugmentation at sites requiring bioremediation. *Rhodococcus jostii* strain RHA1, which was isolated from a γ-hexachlorocyclohexane-contaminated site, can degrade a broad range of organic compounds, such as gasoline components, highly chlorinated PCB, and TCE under aerobic conditions (Hand et al., 2015; Iino et al., 2012; Seto et al., 1995). RHA1 has three linear plasmids, i.e., pRHL1
(1,100 kb), pRHL2 (450 kb), and pRHL3 (300 kb), and dioxygenase genes (bph), which are located on the pRHL1 plasmid and are involved in the initial step of the PCB-degrading pathway (Iwasaki et al., 2007; Yang et al., 2011). Strain RHA1 may be useful for bioaugmentation since no accumulation of toxic intermediates, such as cDCE and VC, was observed during TCE degradation by this strain.

To control field-scale bioaugmentation and to select adequate bioaugmentation methods, it is necessary to assess the conditions for pollutant degradation by microorganisms and the ecological effects of environmental factors, including microbial communities. In particular, there is concern that the injection of microorganisms may cause changes in the microbial community balance, resulting in increased numbers of harmful microorganisms and, thereby, affecting human health and water quality. In this study, we attempted multi-omics analysis to assess the ecological impact at the bioaugmentation site. Multi-omics approaches provide comprehensive information on the microbial community, and metatranscriptomic approaches provide information to predict the degradation mechanism of pollutants according to different degraders. Our findings demonstrated that the inoculated strain contributed to TCE/cDCE degradation in groundwater, but also caused an irreversible shift in the microbial community structure.

MATERIALS AND METHODS

Site characterization

The bioaugmentation site was located in a field of a chemical company, which was contaminated with TCE/cDCE/VC. Figure S1 shows a site map of the wells, including the two injection wells (E1 and E4). The test site was surrounded by embedded steel sheet piles enclosing an area of 2.0 m × 2.0 m arranged to prevent water inflow or outflow. The steel sheet
pile was installed one month before the bioaugmentation experiment and pumped 1 kL water.

E1 mostly consisted of a similar soil matrix, containing concrete, brick (GL 0.0 m to -2.5 m), fine sand (GL -2.5 m to -5.0 m), medium sand (GL -5.0 m to -7.0 m), gravel (GL -7.0 m to -8.0 m), coarse sand (GL-8.0 m to -9.0 m), and silt (below -9.0 m). The E4 well was the control well.

**Cell culture and injection of cells into the well**

RHA1 cells were grown using a fermentation tank under aeration-agitation conditions. RHA1 was incubated at 30°C for 64 h in 150 L LB medium with defoaming agent. After cultivation, RHA1 was centrifuged for 10 min at 20°C and 5,000 rpm, and the collected pellet was washed with 10 mM potassium phosphate buffer (pH 7.5). The washed RHA1 was suspended in 7 L of the same buffer, and this mixture was used as a cell suspension (1.0 ×10^{14} cells). The cell suspension was suspended in 1 kL aeration water and injected into the E1 well. In addition, aeration water was injected into the control well with an injection rate of 2.0 kL/h.

**Groundwater collection**

Groundwater samples (2 L) were collected in plastic bottles from the E1 and E4 wells at 30 days before injection and on days 0, 1, 6, 19, and 54 after injection of RHA1 cell aeration water. Collected samples were centrifuged for 10 min at 4°C and 10,000 rpm and then stored at -80°C until subsequent analysis.

**Analytical procedures**

The analytical procedures for determination of physicochemical parameters were carried out as described by Miura et al. (2015). The 16S rRNA gene copy numbers were analyzed by real-time PCR as described by Ritalahti et al. (2006). The copy number of the RHA1 etbAc gene was determined by quantitative PCR (qPCR) using a Light Cycler 96 (Roche, Basel, Switzerland). The primers were 052-Fw (5'-CGTTTGTGCTGTGGATGAA-3') and 143-Rv (5'-TCGCCGACCCGGTATG-3'). The probe was
(5’-FAM-AAAACCTgTCgCTgAACCCACC-TAMRA-3’). Each 20-µL reaction mixture contained 6 µL sterilized deionized water, 10 µL THUNDERBIRD Prove qPCR Mix (TOYOBO, Osaka, Japan), 1 µL 052-Fw primer (4 µM), 1 µL 143-Rv primers (4 µM), 1 µL probe (4 µM), and 1 µL extracted DNA. PCR was conducted by incubating the samples at 95°C for 60 s, followed by 50 cycles of 95°C for 15 s and 52°C for 60 s.

**DNA, RNA, and cDNA preparation**

Pellets (0.5 g) were suspended in 200 µL of sterilized water, and the mixture was then transferred to Lysing Matrix E (MP-Biomedicals, Santa Ana, CA, USA). DNA was extracted using a Fast DNA Spin Kit for soil (MP-Biomedicals) according to the manufacturer’s instructions. DNA was quantified using a Qubit dsDNA HS assay kit (Invitrogen, Grand Island, NY, USA) on a Qubit 2.0 Fluorometer (Invitrogen). RNA was extracted using a Fast RNA Spin Kit for soil (MP-Biomedicals) according to the manufacturer’s instructions, and RNA was then purified using an RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. For removal of DNA, RNA samples were treated using DNase (Takara Bio, Shiga, Japan) and extracted by ethanol precipitation. RNA was quantified using a Qubit dsRNA HS assay kit (Invitrogen) and Agilent RNA 6000 Pico (Agilent, Palo Alto, CA, USA). RNA was purified using Ribozero (Illumina, Inc., San Diego, CA, USA) and was quantified using an Agilent RNA 6000 Pico (Agilent).

**Metagenome sequencing and analysis**

For metagenome sequencing, the libraries were constructed using a TruSeq DNA Sample Prep Kit v2 (Illumina, Inc.). These libraries, prepared with a fragment length of about 325 bp, were sequenced with a HiSeq 1000 sequencer using TruSeq SBS Kit v3-HS (Illumina, Inc.). DNA sequences were annotated with MetaGenomics Rapid Annotation using Subsystem Technology (MG-RAST) pipeline version 4.0 with a minimum alignment length of 15 bp, minimum identity
of 60%, and maximum E-value cut off of $1.0 \times 10^{-5}$ (Meyer et al., 2008). Three million sequence reads were used for the analysis of each sample. Taxonomic analysis was conducted with a normalized abundance of sequences matched to the M5NR database. Functional analysis was determined at levels 1, 2, and 3 by matching normalized sequence abundance to a given subsystem. Principal coordinates analysis (PCoA) and alpha-diversity were analyzed by MG-RAST.

**Transcriptome sequencing and analysis**

For cDNA preparation, a TruSeq DNA Sample Prep Kit v2-setA (Illumina, Inc.) and SuperScript III Reverse Transcriptase (Invitrogen) were used to synthesize cDNA, following the manufacturer’s instructions. Sequencing of the synthesized cDNA sequence was performed by Hiseq1000 (Illumina, Inc.) with a fragment length of about 325 bp. Data analysis of the transcriptome was performed using R (ver.3.3.2). Mapping of reads to the RHA1 genome (NC_008268.1, NC_008269.1, NC_008270.1, and NC_008271.1), *Geobacter lovleyi* SZ genome (NC_01814.1 and NC_010815.1), and *Dehalococcoides mccrartyi* 195 genome (NC_002936.3) was performed using the Bioconductor package QuasR allowing 2 bp mismatches (Langmead et al., 2009). Mapped read counts per gene were obtained using the qCount function of QuasR and annotation data formatted by the Bioconductor package rtracklayer (Lawrence et al., 2009; Love et al., 2014). Differential expression analysis was performed using Bioconductor package DESeq2 (Langmead et al., 2009; Lawrence et al., 2009; Love et al., 2014). For hierarchical clustering, we normalized read count data across samples using the varianceStabilizingTransformation function of DESeq2 prior to clustering (Langmead et al., 2009; Lawrence et al., 2009; Love et al., 2014).

**Nucleotide sequence accession numbers**
The metagenome and metatranscriptome sequences were deposited in the DDBJ database under accession numbers DRA005882 and DRA006583.

RESULTS

Analysis of parameters after treatment of the contaminated site

To evaluate the effects of RHA1 injection, the E1 and E4 wells were monitored between days -30 and 54 (Table 1). The pH value was 6.2 at day -30 in the E1 well and ranged from 6.8 to 7.2 after RHA1 injection. The oxidation-reduction potential (ORP) was 98 mV at day -30 and increased to 181 mV at day 0, then decreased to -158 mV at day 54. The electrical conductivity (EC) value was 99 mV at day -30 and then decreased to 25 mV at day 0 and increased to 80 mV at day 54. The dissolved oxygen (DO) value was 0.0 mg/L at day -30 and increased to 5.8 mg/L at day 0. The DO value in E1 well decreased significantly to 0.3 mg/mL at day 1 and then to below the detection limit between days 6 and 54. The dissolved organic carbon (DOC) value was 5.7 mg/mL at day -30 and increased to 6.1 mg/mL at day 54. The concentration of total iron was 10.4 mg/L at day -30 and then decreased to 0.2 and 0.5 mg/L at days 0 and 1, respectively. Moreover, the concentrations of total iron were increased to 11.0, 15.5, and 18.9 mg/L at days 6, 19, and 54, respectively. At day 54, the concentrations of o-phosphoric acid and ammonia nitrogen were higher than before treatment.

The time course of chlorinated ethylenes levels in the E1 well is shown in Fig. 1A–C. In the E1 well, the levels of TCE and cDCE decreased to 69.2% and 71.4% at day 54. Subsequently, the concentration of VC was significantly increased from $2.0 \times 10^{-3}$ mg/L at day 6 to $2.7 \times 10^{-2}$ mg/L at day 54.

In the E4 well, the pH values were 6.3, 6.7, 6.6, 6.4, 6.4, and 6.4 at days -30, 0, 1, 6,
The ORP value was 95 mV at day -30 increased to 230 mV at day 0 and then decreased to 102 mV at day 54. The EC and DOC values were lower at day 54 than before treatment. The DO value was 0.0 mg/L at day -30, increased to 7.0 mg/L at day 0, and gradually decreased to 1.4, 3.6, 5.3, and 13.1 mg/L at days 1, 6, 19, and 54, respectively. The concentration of total iron was 13.5 mg/L at day -30, decreased significantly to 0.6 mg/L at day 0, and then gradually increased to 1.9 × 10^4 copies/mL at day 54. No significant changes were observed in the concentration of o-phosphoric acid and ammonia nitrogen compared with that before treatment at day 54. The level of TCE decreased to 60.0% at day 54, and that of cDCE increased from 0.3 mg/L at day -30 to 0.4 mg/L at day 54. The concentration of VC increased from 1.0 × 10^{-3} mg/L at day -30 to 2.0 × 10^{-3} mg/L at day 54 (Fig. 1A–C).

After injection of RHA1 culture, the total number of 16S rRNA genes was 1.5 × 10^7 copies/mL at day 0 in the E1 well (Fig. 2A). The total number of 16S rRNA genes then gradually decreased to 7.6 × 10^6 copies/mL at day 54. The time course of changes in the RHA1 population in the E1 well is shown in Fig. 2B. The RHA1 population increased from 0.0 copies/mL at day -30 to 2.2 × 10^7 copies/mL at day 0 and then gradually decreased thereafter to 1.9 × 10^4 copies/mL at day 54. In contrast, the population of Dehalococcoides was increased from 7.8 copies/mL at day -30 to 4.8 × 10^3 copies/mL at day 54 (Fig. 2C).

In the E4 well, the total number of 16S rRNA genes was 5.8 × 10^5 copies/mL at day -30, and this value gradually decreased to 3.2 × 10^5, 1.1 × 10^4, 8.2 × 10^4, 8.3 × 10^3, and 2.9 × 10^3 copies/mL at days 0, 1, 6, 19, and 54, respectively (Fig. 2A). An RHA1 population was detected in the samples collected from days -30 to 19 by qPCR; however, no RHA1 population was detected at day 54. In addition, the Dehalococcoides population was barely detectable from days -30 to 6 by qPCR, and no Dehalococcoides were detected from days 19 to 54 (Fig. 2C).
Bacterial community analysis by whole-genome shotgun sequencing

In the E1 well, metagenomic analysis revealed that the phylum Proteobacteria was dominant before injection of RHA1 cells (at day -30; 85.8%), and the class Betaproteobacteria (73.9%) accounted for over half of the phylum Proteobacteria. At the genus level, Acidovorax (14.5%), Polaromonas (9.6%), and Albidiferax (9.2%; Fig. 3A and B, Tables S1, S2A, and S2B) were present. After RHA1 injection, the phylum Actinobacteria was dominant throughout the monitoring period (84.1% at day 0, 72.5% at day 1, 71.3% at day 6, and 40.3% at day 19). At the genus level, Rhodococcus was dominant, and at the phylum level Actinobacteria accounted for 76.6%, 65.8%, 64.4%, and 34.9% at days 0, 1, 6, and 19, respectively (Table S3). In addition, the population of the phylum Firmicutes increased to 13.8% and 18.9% at days 0 and 1, respectively, and then decreased to 5.0% and 8.7% at days 6 and 19, respectively. At the genus level, Bacillus was the most abundant in the phylum Firmicutes at days 0 and 1. Although the genus Geobacter decreased from 1.5% at day -30 to 0.0% at day 0, the population of Geobacter increased to 5.8% and 22.6% at days 6 and 19, respectively.

PCoA of the bacterial community structures

PCoA revealed that the microbial community in the E1 well was significantly changed after RHA1 injection (Fig. S2). Indeed, this analysis revealed that the microbial communities were different between days -30 and 19. Additionally, α-diversity was 246 at day -30, 6 at day 0, 11 at day 1, 14 at day 6, and 61 at day 19 (Table S4) and decreased after RHA1 injection, although the level subsequently increased over time.

Functional profile of the bacterial community

To determine the genetic potential of the microbial communities of TCE/cDCE-contaminated groundwater and their adaptive features with regard to the biodegradation of xenobiotic compounds, functional annotations were carried out according to KEGG hits, and we assigned
predicted functions to the metagenome shotgun sequences. After injection of the RHA1 culture in the remediation site at the E1 well, the portion of functional genes involved in the metabolism of aromatic compounds increased from 1.8% at day -30 to 7.0% at day 0 (Table 2). The portion of functional genes involved in dormancy and sporulation also increased from 0.1% at day -30 to 0.6% at day 0. These results indicated that the functional profile of the bacterial community was affected by RHA1 culture injection.

Gene expression of aromatic compound-degrading enzymes

To test whether the strain RHA1, inoculated as a bio-degrader, was physiologically active in the E1 well, we extracted the total RNA from contaminated soils, and the expression levels of genes on the RHA1 plasmids pRHL1, pRHL2, and pRHL3 were estimated by transcriptome sequencing (RNA-seq; Fig. 4). We focused on these three plasmids since the complete set of genes responsible for aromatic compound degradation was located on the plasmids in RHA1. To screen genes that showed changes in expression levels after inoculation of RHA1, we initially conducted pairwise comparisons in data for days 0 and 1. Twenty-seven genes showed relatively low q.values (false discovery data) of less than 0.1. Normalized read counts for these 27 genes were obtained from four time points, and their expression values were shown as Z scores in the heatmap (Fig. 4). These data suggested that all 27 genes showed decreased expression over time. Among the selected 27 genes, bphAa (RS35885), bphAb (RS35880), bphAc (RS35875), and bphAd (RS35870) were estimated to be involved in the degradation of TCE/cDCE. The gradually decreased expression of these genes was consistent with the decrease in TCE/cDCE in the environment. The inoculated strain was expected to be physiologically active in producing degradation enzymes, but showed a decreased degradation activity relatively rapidly.

DISCUSSION
In this study, we performed multi-omics analysis to assess the ecological impact at the bioaugmentation site. At the initial stage of TCE/cDCE degradation, RHA1 was the dominant bacterium in the E1 well after RHA1 injection. Moreover, gene expression of biphenyl-degrading enzymes in RHA1 was detected immediately after RHA1 injection. As a result of metagenome analysis, the proportion of *Rhodococcus*, including RHA1 in the groundwater, at day 0 increased by 76.5% when compared with that at days -30 and 0, making RHA1 the dominant species. In addition, the proportion of *Bacillus* also increased by 8.5% when comparing days -30 and 0. The populations of other aerobic microorganisms, such as *Mycobacterium*, *Geobacillus*, and *Paenibacillus*, also increased at the injection site. At the E4 well, changes in TCE levels may have been caused by temporary dilution with the aeration water and natural attenuation by activated native bacteria, which can degrade TCE under aerated conditions. However, TCE-degrading bacteria could not be identified from metagenome data from the E4 well. Previous studies revealed that *Mycobacterium vaccae* JOB 5 and *Geobacillus* sp. strain FW23 are able to degrade TCE and their metabolites (Dey and Roy, 2009; Wackett, 1989; Pore, 2014). Another study reported that *Paenibacillus kobensis*, *Paenibacillus curdlanolyticus*, and *Paenibacillus wynnii* can grow even in TCE-contaminated soil. These results suggested that the other aerobic bacteria, as well as strain RHA1, may be involved in TCE degradation at the injection site (Mera and Iwasaki, 2007). These findings provide a new point of view for the selection of bioremediation methods and the evaluation of the effects on the microbial environment during bioremediation.

Degradation of TCE/cDCE by strain RHA1 was not observed after day 6 since this bacterium completely consumed the oxygen required for growth and degradation of pollutants. In addition, we verified gene expression in RHA1 using metatranscriptomic analysis and observed high expression in 27 oxygenase genes at day 0. Degradation of TCE/cDCE by
aerobic bacteria resulted in the generation of formic acid and carbon dioxide via TCE/cDCE epoxide (Suttinun et al., 2013). Previous studies reported that monoxygenase and dioxygenase are involved in the production of TCE/cDCE epoxide as a TCE/cDCE metabolite (Suttinun et al., 2013; Furukawa, 2006). For example, in *Xanthobacter* strain Py2, alkene monoxygenase and epoxidase are involved in the production of TCE/cDCE epoxide (Ensign, 1996). Furthermore, in *R. erythropolis* BD 2 and *Rhodococcus* sp. L 4, isopropyl benzene/toluene dioxygenase has been reported to be involved in the degradation of TCE (Suttinun et al., 2013). In the case of strain RHA1, genes involved in producing TCE/cDCE epoxide have not been reported; however, propane/alkane monoxygenase or biphenyl dioxygenase of strain RHA1 was expressed in the presence of triclosan as a substrate (Lee and Chu, 2013). Thus, aromatic compound and alkane oxygenases may be responsible for the initial reaction in the aerobic degradation of TCE/cDCE. Although 203 oxygenases were annotated in the genome of strain RHA1, seven oxygenase-related genes, including biphenyl 2,3-dioxygenase subunit genes, *bphAa* (RS35885), *bphAb* (RS35880), *bphAc* (RS35875), *bphAd* (RS35870), and *etbAc* (non-heme iron oxygenase ferredoxin subunit; RS41430), and 2,3-dihydroxybiphenyl 1,2-dioxygenase genes, *bphCI* (RS35865) and *etbC* (RS41380), were found to be highly expressed at day 0 in this study (McLeod et al., 2006). Therefore, these results suggested that some of these highly expressed genes are involved in the degradation of TCE/cDCE.

On day 6 after the beginning of TCE/cDCE bioremediation, TCE/cDCE may be converted to VC by anaerobic bacteria, i.e., *Geobacter* and *Dehalococcoides*, which were increased in the injection site. Strain RHA1 was related to the consumption of DO, caused decreased ORP levels, and created the observed anaerobic conditions. Moreover, increased DOC, *o*-phosphoric acid, ammonia nitrogen, and EC were observed after day 6, and these changes were expected to be the cause of dissolution of the cellular components from strain
RHA1 in the groundwater. Therefore, the chemical conditions at the injection well have been changed to enhance the growth of, and dechlorination by, Geobacter and Dehalococcoides. The concentration of VC significantly increased in the injection well from days 6-54 in this study, and VC was accumulated in the well around 27 times before treatment. Moreover, a rebound in TCE/cDCE at day 6 was observed, resulting in the creation of the conditions for easier accumulation of VC. On the basis of the microbial community analysis, anaerobic bacteria, i.e., Geobacter and Dehalococcoides, were detected at the injection site at days 6 and 19 after treatment. From these results, it was speculated that Geobacter was related to the conversion of TCE to cDCE and that Dehalococcoides relied on the degradation of cDCE to VC as a metabolite of TCE at the injection site. In contrast, the E4 well did not become anaerobic because there was no oxygen consumption by RHA1. Therefore, cDCE degradation was not observed at the E4 well. A previous study reported that accumulation of VC was observed during the bioaugmentation of TCE using Dehalococcoides spp., Dehalobacter sp., and G. lovleyi strain SZ (Amos et al., 2009). According to these results, key dechlorinators of TCE/cDCE were mainly aerobic bacteria, such as RHA1 and Bacillus, until day 1, and the key dechlorinators then shifted to anaerobic bacteria, such as Geobacter and Dehalococcoides, beginning on day 6 in the injection well.

In this study, the microbial communities at the injection site were disrupted by the injection of RHA1 cultures. A previous study also reported that the microbial composition in the TCE bioremediation site was significantly different before and after treatment using Dehalococcoides strains FL 2, BAV 1, and GT with acetic acid as an electron accepter (Adetutu et al., 2015). Furthermore, succession of the microbial composition at the remediation site showed that ε-Proteobacteria and γ-Proteobacteria, which were not injected, were dominant species in the bioremediation site during bioremediation. In this study, PCoA revealed that
succession of the microbial community was also observed at the injection site during
bioremediation after treatment. These results suggested that injection of the culture of the
degrading strain led to disruption of the microbial community and that the succession of the
microbial community composition occurred after injection of the culture.

In summary, in this study, we used multi-omics analysis to analyze the roles of
degrading bacteria in the metabolism of TCE/cDCE at bioremediation sites. The advantage of
metagenomic analysis is that the bias of culturing microorganisms from the environmental
samples can be excluded, even though the population of microorganism was very low.
Metatranscriptomic analysis can analyze the transcription of an enormous number of genes
present in the environment. Thus, multi-omics analysis provides useful information for
comprehensively analyzing microbial community succession and predicting the degradation
pathway for contaminated compounds at bioremediation sites.

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References

intrinsic microbial degradative potential for field-based in situ dechlorination of

distributions of *Geobacter lovleyi* and *Dehalococcoides* spp. during bioenhanced


Heron, G., Lachance, J., and Baker, R. (2013) Removal of PCE DNAPL from tight clays using


metagenomes. *BMC Bioinform.*, 9, 386.


Figure Legends

Fig. 1. Time course of contamination levels of TCE, cDCE, and VC at E1 and E4 wells. (A) TCE, (B) cDCE, (C) VC. Closed circles, E1 well; open squares, E4 well.

Fig. 2. Changes in the (A) total number of bacteria, (B) the RHA1 population, and (C) the *Dehalococcoides* population, during the bioaugmentation period at the E1 and E4 wells. Closed circles, E1 well; open squares, E4 well.

Fig. 3. The abundance of bacterial groups at the (A) phylum, and (B) genus, levels in pre- and post-treatment samples from the E1 well.

Fig. 4. Heatmap of genes involved in degradation of TCE/cDCE.

Fig. S1. Schematic representation of the E1 and E4 wells.

Fig. S2. PCoA based on metagenome analysis using MG-RAST at the E1 well.
Figure 1

- **A**: Concentration [mg/L] vs. Time [days]
  - Concentration values range from 0 to 0.015 mg/L.
  - Time values range from -30 to 54 days.

- **B**: Concentration [mg/L] vs. Time [days]
  - Concentration values range from 0 to 0.04 mg/L.
  - Time values range from -30 to 54 days.

- **C**: Concentration [mg/L] vs. Time [days]
  - Concentration values range from 0 to 0.03 mg/L.
  - Time values range from -30 to 54 days.
Figure 2

A

B

C

Number of cells
[16S rRNA gene copies/mL]

Number of cells
[ethAc gene copies/mL]

Number of cells
[16S rRNA gene copies/mL]

Time [days]
Figure 3

Bacterial abundance

<table>
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<tr>
<th>Time [days]</th>
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Bacterial abundance

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</table>
Figure 4

Color Key

-1 0 1
Row Z-Score

Day 0 1 6 19

RS41325  AMP-dependent synthetase
RS41405  LLM class F420-dependent oxidoreductase
RS41320  enoyl-CoA hydratase
RS41430  non-heme iron oxygenase ferredoxin subunit (etbAc)
RS41465  sterol transfer protein
RS41340  ferredoxin reductase
RS41345  3-(cis-5,6-dihydroxycyclohexa-1,3-dien-1-yl) propanoate dehydrogenase
RS41400  ketosteroid isomerase-related protein
RS41390  2-oxo-hepta-3-ene-1,7-dioic acid hydratase
RS41395  cleaves 5-dehydro-4-deoxy-glucarate and 2-dehydro-3-deoxy-D-glucarate
RS41310  acetalddehyde dehydrogenase
RS44830  hypothetical protein
RS35830  quinone oxidoreductase
RS35880  biphenyl 2,3-dioxygenase subunit beta (bphAb)
RS41385  2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase
RS35860  3-(cis-5,6-dihydroxycyclohexa-1,3-dien-1-yl) propanoate dehydrogenase
RS35890  2-hydroxy-6-oxo-2,4-heptadienoate hydrolase
RS35885  biphenyl 2,3-dioxygenase subunit alpha (bphAa)
RS35865  2,3-dihydroxybiphenyl 1,2-dioxygenase (bphC1)
RS41380  2,3-dihydroxybiphenyl 1,2-dioxygenase (etbC)
RS41435  2-hydroxy-6-oxo-2,4-heptadienoate hydrolase
RS35870  biphenyl 2,3-dioxygenase ferredoxin reductase component (bphAd)
RS41420  ribosomal subunit interface protein
RS41370  ribosomal subunit interface protein
RS35840  AMP-dependent synthetase
RS35825  hypothetical protein
Table 1. Geochemical characteristics of groundwater at E1 and E4 wells.

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<th>Well</th>
<th>Days after injection</th>
<th>Temp [°C]</th>
<th>pH</th>
<th>ORP [mV]</th>
<th>EC [mV]</th>
<th>DO [mg/L]</th>
<th>DOC [mg/L]</th>
<th>Total iron [mg/L]</th>
<th>o-phosphoric acid [mg/L]</th>
<th>Ammonia nitrogen [mg/L]</th>
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Table 2. Functional analysis (subsystem/level 1) based on metagenome analysis using MG-RAST at E1 well.

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<th>Abundance [%]</th>
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<td>Carbohydrates</td>
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<tr>
<td>Clustering-based subsystems</td>
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<td>Cofactors, vitamins, prosthetic groups, pigments</td>
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<tr>
<td>DNA metabolism</td>
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<td>Dormancy and sporulation</td>
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<td>Metabolism of aromatic compounds</td>
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<td>Miscellaneous</td>
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<td>Category</td>
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<td>Motility and chemotaxis</td>
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<td>Nitrogen metabolism</td>
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<tr>
<td>Nucleosides and nucleotides</td>
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<tr>
<td>Phages, prophages, transposable elements, plasmids</td>
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<td>Sulfur metabolism</td>
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<tr>
<td>Virulence, disease and defense</td>
<td>3.8</td>
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